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TITLE: Persistently Elevated Somatic Mutation as a Biomarker for Clinically Relevant Exposures in GWI

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14. ABSTRACT
Gulf War Illness (GWI) consists of a set of debilitating symptoms that have been associated with deployment to the Persian Gulf theatre of war, either in 1990/91 in Kuwait or 2003-11 in Iraq. There is general agreement that physical exposures play an important role in the etiology of this disease (or diseases), yet studies to identify a single major causative exposure have been largely unproductive.

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None provided

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Introduction

Veterans who served in the Gulf war report debilitating health symptoms 2-3x more frequently than military personnel who were not deployed to the Gulf. These symptoms are multi-system and non-specific, involving fatigue, headache, memory issues, sleep disorders and musculoskeletal pain. Gulf war illness (GWI) is a life-altering disease presumably caused by exposures to radiation and/or chemicals. However, since some, but not all personnel manifest this disease, there may be an additional genetic component that increases vulnerability to such exposures. We hypothesize that our approach to environmentally-induced carcinogenesis, to measure the total effect of all genotoxic exposures, as modified by the genetic susceptibility of each exposed individual, can be translated successfully to a study of GWI. From our previous studies, it is clear that genotoxic exposures can induce both short-term and long-term effects, with the long-term effects associated with mutagenesis of the stem cell compartment. Stem cell mutagenicity, as demonstrated by persistent elevations in blood-based somatic mutation frequencies, would be expected to result in pleiotropic premature aging effects that could manifest as non-specific GWI. We therefore propose to directly measure somatic mutation frequencies in symptomatic and asymptomatic Gulf war veterans and controls to determine whether elevated somatic mutation is indicative of disease or disease severity. In a subset of subjects with elevated somatic mutation we will also directly measure DNA repair capacity, to determine whether genetic predisposition is an important element in determining who will manifest clinically relevant symptoms. Identifying the basis of genetic predisposition would also allow for the sequestering of "high-risk" personnel from exposures more likely to produce clinical disease in future deployments.

Keywords

Genotoxic exposure, genetic predisposition, somatic mutation, DNA repair, stem cells

Accomplishments

What were the major goals of the project?

I. Determine whether symptomatic Gulf war veterans have persistently elevated levels of bone marrow somatic mutation.

II. Determine whether there is an association between elevated somatic mutations frequencies and the number or severity of symptoms in GWI.

III. Determine whether symptomatic Gulf war veterans with elevated somatic mutation frequencies are functional deficient for DNA nucleotide excision repair.

What was accomplished under these goals?

Goal I:

Adaptation of the GPA somatic mutation assay to new flow cytometry platform

Although Dr. Grant has published 25 papers on the GPA somatic mutation assay, it requires commercial reagents and a commercial flow cytometry platform, and has had to be reinvented several times over the last 30 years. We had not re-established the assay since moving to Nova Southeastern University, and were surprised to find that we had to find new vendors for critical reagents and adapt the technique to new technology in the form of the BD Accuri C6 flow cytometer. In this, we are indebted to Ph.D. student Omar Ibrahim, whose thesis project involves flow cytometric characterization of stem cells. We have developed a new protocol that yields somatic mutation frequency results indistinguishable from those of the previous "DR6" version of the assay on historical controls. We are still investigating whether new functionalities can improve the assay (in the analysis mode, not performance mode), then it is likely that we will publish the new protocol to make it available to the research and public health communities.

Ms. Sveiven participated in the development of the new version of the assay, which required her to learn about flow cytometry and proper use and maintenance of flow cytometric equipment. This knowledge has now been extended to Ms. Foley.

Blood sampling of GWI patients and controls

In order to redevelop the GPA assay, and to establish a bank of controls for each run (see below), we have sampled 8 local volunteers form the Grant/Latimer and Klimas groups. We have received 17 experimental blood samples, unidentified as to patient or control. As we fear this rate of accumulation will not allow us to enroll our required subjects, we have adjusted personnel to have an ongoing, active presence in the Klimas group.

Performance of the GPA somatic mutation and UDS DNA repair assays on GWI patients and controls

As every "run" of the GPA assay requires the concurrent analysis of at least 3 controls, we bundle samples in order to maximize each set of controls. This can be done because fresh samples can be archived for a matter of weeks before analysis and we also routinely archive fixed and frozen samples that are usable indefinitely. We bundle samples by their GPA phenotype (MM, MN or NN) because what is a main peak in one sample may be the experiment endpoint in the next, and, when our mutant frequencies are based on a few aberrant cells per million, we cannot risk cells from one sample retained in the cytometer showing up as mutant cells in the next analysis. Thus, of the 17 samples collected thus far, we have processed all of them, but only 13 have been analyzed. As mentioned earlier, we have also determined to perform the UDS assay on a larger subset of subjects, and thus far that has tranbslkated to 6 UDS assays, along with several local controls (since we had not previously applied the assay to blood lymphocytes here at Nova Southeastern University).

Goal II:

Not active until Year 3: cannot be done until GPA data is broken into cases and controls

Goal III:

Performance of the UDS assay of DNA nucleotide excision repair on GWI patients and controls

The original study design involved performance of a nested study of DNA repair in lymphocytes in subjects (both patients and controls, so that it could be done without breaking anonymization) that exhibited high GPA somatic mutation frequencies. Early analysis of extra data provided by the Klimas group (see next section), suggested that DNA repair alteration might be more pervasive in the GWI population than we had initially hypothesized. We have therefore performed the UDS assay on all samples provided, rather than only a subset. Ms. Sveiven already had experience with the UDS assay working in Dr. Latimer's laboratory, although this was the first application to fresh lymphocytes (rather than cultured cells) performed at Nova Southeastern University. Ms. Foley has now been trained in this technique as well.

Analysis of DNA nucleotide excision repair gene expression in GWI patients and controls

Soon after then announcement of funding, Dr. Lubov Nathanson of Dr. Klimas' group offered to share with us blood-based gene expression microarray data that they had generated on 10 GWI patients and 10 matched controls. The results of interrogating this data set for expression of the 20 most important in the pathway for DNA nucleotide excision repair, as defined by our studies of tissue specificity and breast cancer, showed a significant reduction in expression of this pathway among patients with GWI (Figure 1). We are indebted to former Ph.D. student Homood As Sobeai, whose thesis work involved analysis of microarray data in breast cancer and acute myelogenous leukemia, for this analysis.

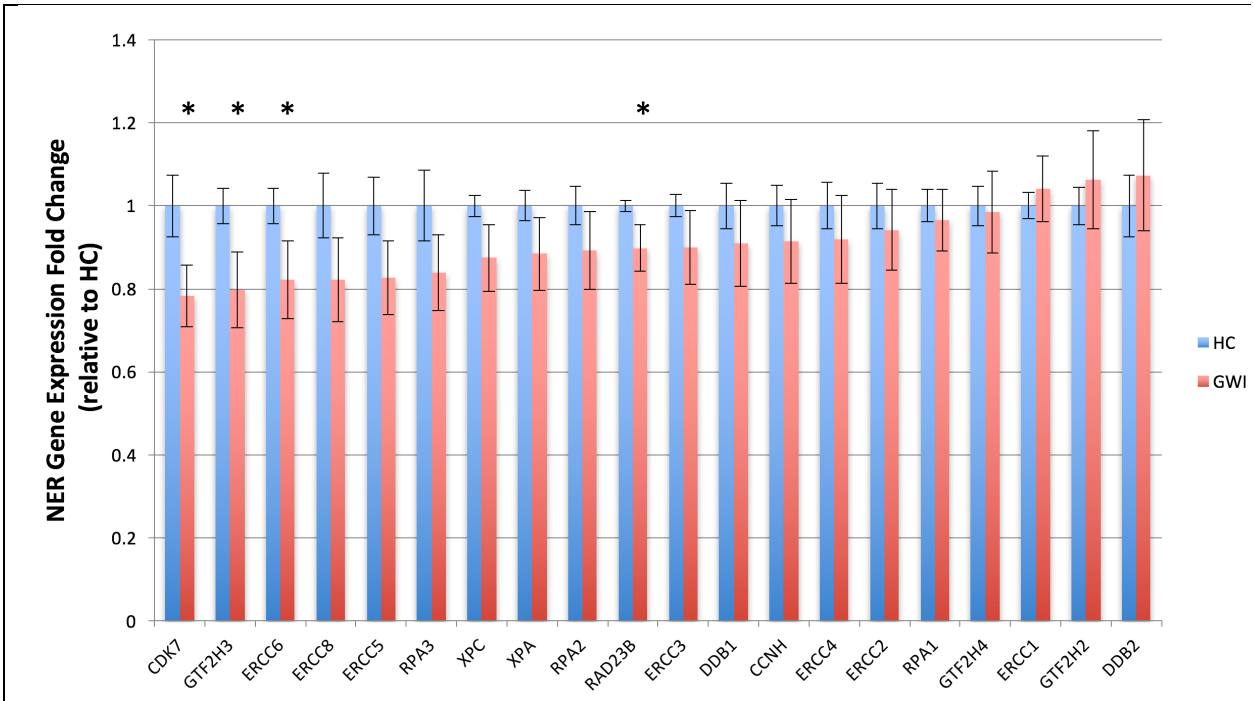

Figure 1. Expression of NER genes in 10 GWI patients (red bars) relative to 10 matched controls (blue bars). 17/20 genes were underexpressed in the patients samples, with the reduction in expression of 4 genes individually statistically significant ($p < 0.05$).

These data demonstrated that gene expression was significantly skewed towards underexpression of the NER pathway in the population of GWI patients ($P = 0.041$, Fisher's exact test). Since gene expression microarray is no longer a cutting-edge technique, however, we are now confirming this observation in an ongoing project capturing RNAseq from patients in our study. This work is being done by Ph.D. student Manasi Pimpley in collaboration with Dr. Nathanson. Ms. Pimpley's thesis work involves RNAseq analysis of breast cancer cell lines and normal breast epithelium.

Performance of the UDS assay on stem and non-stem cells to determine functional capacity of DNA nucleotide excision repair

We have previously characterized some types of genotoxic exposure, particularly the chemotherapeutic agent cis-platinum, as having greater capacity to affect stem cells than other agents. As part of his thesis work on breast cancer, Mr. Ibrahim has flow cytometrically isolated stem and non-stem cells from 3 cell lines derived from different stages of breast cancer (Table 1). In each case, the stem cells had greater DNA repair capacity than the non-stem (somatic) cells. This work may have implications for interpreting long term effects of exposure if somatic effects are transitory and only stem cell effects persist.

Table 1. Nucleotide excision repair capacity in stem cells vs. non-stem cells				
			NER Capacity (relative to Foreskin Fibroblasts; %)	
Breast cancer cell line	Derivation	Proportion Stem Cells (%)[1]	Stem cells	Non stem cells
MDA-MB231	Stage IV	80.7	106.7	64.2
JL-BTL-12	Stage III	82.6	48.6	26.2
JL-BTL-29	Stage II	76.9	44.7	37.7
[1]Defined as CD24-/CD44+/CD49f+				

What opportunities for training and professional development has the project provided?

As described above, Ms. Sveiven has either been initially trained in the techniques required for this project or has adapted her existing skills under the mentorship of Dr. Grant and Latimer. Ms. Foley, who initially filled the "to be named" position, has likewise been trained in these technologies.

As can be seen, Drs. Grant and Latimer are applying many of the techniques from their ongoing collaborations on breast cancer and leukemia to this study on GWI, and this has allowed for the participation of 3 graduate students, Homood As Sobeia, Omar Ibrahim and Manasi Plimpley in aspects of the study, introducing them directly to the subject and showing how their knowledge and skills can be applied in new arenas.

Drs. Grant and Latimer and their students are regular participants in the Seminar series run by Dr. Klimas' Institute for Neuro-Immune Medicine. Dr. Grant has also participated in one local, one national and one international meeting on military health issues and Gulf War illness.

How were the results disseminated to communities of interest?

Although we really only have incidental findings at this point, our ongoing progress is discussed at weekly meetings of the Grant/Latimer lab and monthly meetings that include Dr. Klimas and participating members of her group.

We take every opportunity to discuss our work with the greater scientific community in Nova Southeastern University, soliciting feedback and potential collaboration. An example of this was a recent meeting with scientists from the U.S. Geological Survey, which is located on campus:

Identification and characterization of human environmental exposures. Presented at the Collaborative meeting of Nova Southeastern University Research Institutes and the United

States Geological Survey, Guy Harvey Oceanographic Institute, Nova Southeastern University, November 10, 2016.

As mentioned above, aspects of this work have also been reported at local, national and international meetings in the last year. A poster and a talk were presented at a local meeting at Nova Southeastern University on *Complex Neuro Inflammatory Conditions: GWI and ME/CFS*. Posters were also presented at the national *Military Health System Research Symposium* in Orlando, and the *12th International IACFS/ME Research and Clinical Conference: Emerging Science and Clinical Care* in Fort Lauderdale. Mr. Ibrahim presented some of his stem cell work at the *9th AACR Conference of the Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved* in Fort Lauderdale.

What do you plan to do during the next reporting period to accomplish the goals?

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Impact

What was the impact on the development of the principal discipline(s) of the project?

The most significant impact of the first year's work will probably be in the development of a GPA-based somatic mutation assay compatible with contemporary technology. The GPA assay has been applied in almost 400 scientific papers, but, as mentioned earlier, it requires periodic reinvention, which seems only to occur in a select few labs. With the publication of a revised and updated protocol that can be directly applied in outside labs, this project will have provided for a continuation of the ability to biomonitor genotoxic effects in known and suspected exposed populations, medical patients and patients with genetic susceptibility to cancer and other diseases.

A direct result of providing facile application of a new GPA assay would be proactive monitoring of the populations listed above, rather than passive retrospective analysis. In all of these venues, but significantly including military personnel on active duty, baseline and ongoing monitoring with assays including but not limited to the GPA assay would allow for individualized management of extent of exposure. It should be noted that the GPA assay was originally developed by the DOE for such an application. This was the topic of an editorial published by Dr. Grant in 2012 entitled "Translating mutagenesis into carcinogenesis," although he did not realize at the time that the then-current version of the GPA assay was already obsolete.

Although preliminary, the microarray results shown above are consistent with our initial hypothesis, that soldiers exhibiting GWI are those who are particularly genetically (or epigenetically) susceptible to their exposures. Although this is not a particularly novel concept, the identification of nucleotide excision repair as a mediator of this effect would allow for initial screening and periodic biomonitoring of individuals likely to undergo such exposure.

What was the impact on other disciplines?

The GPA assay has, in the past, been used to detect and quantify both known and unknown genotoxic exposures in studies of environmental, occupational, medical and accidental exposures. It has been used to characterize, and even diagnose, hereditary diseases associated with predisposition to cancer and/or premature aging. It has been used to longitudinally biomonitor cancer patients undergoing genotoxic radio- and/or chemotherapy and victims of radiation accidents and A-bomb survivors. Perhaps because the published protocol required unobtainable reagents and devices, studies using this assay in the U.S. have been declining, although studies in Japan appear to have been maintained and the assay seems to have been embraced in China. Our redevelopment of the assay, upon publication, should reinvigorate its use in these fields, and allow for its application to novel situations, such as the present application to GWI.

What was the impact on technology transfer?

The assay was developed as a method of biologically monitoring workers at risk of genotoxic exposure, and has been validated for radiation workers. Since leaving LLNL, we, and others, have also demonstrated its utility in a number of other occupational settings. The assay has been presented at the R&D facility for Quest diagnostics and an invention disclosure has been filed. We can only hope that a successful application to a high-profile problem, such as GWI, will spur interest in the wider application of this and other blood-based methods of detecting and quantifying genotoxic damage.

What was the impact on society beyond science and technology?

We have presented evidence that a one-time GPA analysis can be highly predictive of cancer risk (if the individual manifests a high “outlier” phenotype, such as the one we are screening for in GWI). It has also been shown to be cumulative dosimeter of genotoxic exposure and damage, integrating host sensitivity factors. Broader application of this and similar analyses would allow for the identification of individuals whose cumulative exposure has pushed them into a category of high risk, detect when they have had a previously unsuspected genotoxic exposure, or quantify the effect of a known exposure, allowing them to avoid further injury.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

Based on the microarray work shown above, we wanted to expand the scope of our study to look for a generalized reduction in DNA repair capacity in symptomatic GW veterans, rather than simply looking for an explanation for high somatic mutation frequency (as established by the GPA assay) outliers.

Actual or anticipated problems or delays and actions or plans to resolve them

We have not been getting access to enough blood samples to reach our sampling goals. We have therefore supported both Ms. Sveiven and Ms. Foley half time on other, related projects, and asked the Klimas group to hire a technician that would be dedicated to representing our interests in sample collection. This hire is pending, and we hope will help us ramp up our sample collection. It is likely that this new hire, who will be part of both the Grant/Latimer and Klimas groups, will also be trained in sample preparation and archiving, and, potentially in the performance of the GPA and UDS assays themselves.

Changes that had a significant impact on expenditures

We had anticipated performing a smaller nested study of UDS analysis based on a more complete study of GPA results. We will have to see whether we can analyze every sample for both assays based on our initial budget.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None

PRODUCTS:

- **Presentations**

Ibrahim, O., Grant, S.G., Myers, N.T., Courtney, A.B., Lalanne, N., and Latimer, J.J. (2016) Analysis of stem cell number and potency in African-American breast tissue. Presented at the *9th AACR Conference of the Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved*, Fort Lauderdale, Florida, September 25–28. *Cancer Epidemiology Biomarkers and Prevention* 26(Supplement): C26.

Grant, S.G., Latimer, J.J., Sveiven, S., Fletcher, M.A., and Klimas, N.G. (2016) Cumulative analysis of total genotoxic exposure and genetic susceptibility to genotoxicity: implications for Gulf War illness. Presented at the meeting on *Complex Neuro Inflammatory Conditions: GWI and ME/CFS*, Nova Southeastern University, Fort Lauderdale, Florida, October 26.

Grant, S.G., Latimer, J.J., Sveiven, S., Fletcher, M.A., and Klimas, N.G. (2016) Cancer as a paradigm for the delayed clinical effects of environmental and occupational exposures: implications for Gulf War illness. Presented at the meeting on *Complex Neuro Inflammatory Conditions: GWI and ME/CFS*, Nova Southeastern University, Fort Lauderdale, Florida, October 26.

Grant, S.G., Latimer, J.J., Sveiven, S., Fletcher, M.A., and Klimas, N.G. (2016) Persistently elevated bone marrow somatic mutation as a biomarker of clinically relevant exposures in Gulf War Illness. Presented at the *12th International IACFS/ME Research and Clinical Conference: Emerging Science and Clinical Care*, Fort Lauderdale, Florida, October 27–30.

Grant, S.G., Latimer, J.J., Nathanson, L., As Sobeai, H., Sveiven, S., Fletcher, M.A., and Klimas, N.G. (2017) Analysis of gene-environmental interactions in mixed military exposures. Presented at the *2017 Military Health System Research Symposium*, Kissimmee, Florida, August 27–39.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	*Stephen Grant, Ph.D.*
Project Role:	*Principle Investigator*
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Grant oversees the entire project. He works with Drs. Klimas and Fletcher to obtain blood samples and with Dr. Latimer to coordinate the performance of the UDS assay. In this first year, he has performed the GPA assay and trained Ms. Sveiven and Foley on this technique.*

Name:	*Jean J. Latimer, Ph.D.*
Project Role:	*Co-Investigator*
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Latimer provides input and expertise for the UDS assay.*

Name:	*Nancy Klimas, Ph.D.*
Project Role:	*Co-Investigator*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Klimas provides the samples to our lab from the Miami facility.*

Name:	*Mary Ann Fletcher, Ph.D.*
Project Role:	*Principle Investigator*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Klimas provides the samples to our lab from the Miami facility.*

Name:	*Patrick Hardigan, Ph.D.*

Project Role:	*Statistician*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Hardigan has consulted with our lab on the data in order to determine the next best steps.*

Name:	*Stefanie Sveiven*
Project Role:	*Research Assistant I*
Nearest person month worked:	*7*
Contribution to Project:	*Ms. Sveiven prepares the samples for RNA, spheres for GPA, cultures for the UDS assay. She runs the UDS assay and analyzes the UDS data. She also assists Dr. Grant in running the GPA assay. She is training Ms. =Foley in the performance of the UDS assay.*

Name:	*Megan Foley*
Project Role:	*Research Assistant I*
Nearest person month worked:	*1*
Contribution to Project:	*Ms. Foley assists Dr. Grant in running the GPA assay. She is learning how to process and archive blood samples, and how to perform the UDS assay.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The PI, Dr. Grant, is also the Director of a large, multi-year, environmental safety training program funded by the National Institute of Environmental Health Sciences, called Project SEAMIST. He recently received funding for a related program from the Occupational Health and Safety Administration, Project LA BRUMA, under the Susan Harwood Training Program, to develop a similar curriculum in Spanish and provide training in that language where appropriate in South Florida. This is a 1-year grant providing for 10% of Dr. Grant's effort. It is expected that these Spanish-language trainings will be folded into the larger program in subsequent years. This has not affected Dr. Grant's effort on the present grant.

No other senior/key personnel report changes in their support.

What other organizations were involved as partners?

Dr. Klimas" group is recruiting patients and controls through two additional partnering institutions:

- **Organization Name:** Miami VAMC
- **Location of Organization:** Miami, FL
- **Partner's contribution to the projec**t: collaboration

- **Organization Name:** Boston University (Dr. Kim Sullivan)
- **Location of Organization:** Boston, MA
- **Partner's contribution to the projec**t: collaboration